Title
Cryopreservation of minced pulp tissue and its scope in Regenerative Endodontic

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Publication Date
2022

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Cryopreservation of minced pulp tissue and its scope in Regenerative Endodontics

A thesis submitted in partial satisfaction

of the requirements of the degree Master of Science

in Oral Biology

by

Dhruvi Pravinchandra Patel

2022

ABSTRACT OF THE THESIS

Cryopreservation of minced pulp tissue and its scope in Regenerative Endodontics

by

Dhruvi Pravinchandra Patel

Master of Science in Oral Biology

University of California, Los Angeles, 2022

Professor Mo K. Kang, Chair

Direct pulp tissue grafting has been proposed in the past to void the tedious task of laboratory culturing. Migration of cells from dental pulp tissue explantation have shown multi differentiation potential in vitro like DPSC. These cells were named MP-MSC (minced pulp derived mesenchymal stem cells) by our group. Establishing that intact pulp tissue can be a potential source of dental pulp derived stem cells, we further wanted to investigate the possibility of pulp tissue banking. Cord tissue banking has gained popularity and success in the past decade

and has shown to be successful in medicine. Our study demonstrated that cryopreserved pulp tissue has a potential to sustain viable cells. Frozen tissue derived mesenchymal stem cells additionally showed that they can maintain the osteogenic and odontogenic differentiation potential in vitro like unfrozen tissue minced pulp tissue via expression of various markers such as ALP, OCN, Collagen 1 and 3 markers suggesting, that they maintained the differentiation potential after cryopreservation. Ex vivo tissue engineering showed migration of cells and attachment to dentin surface in presence of scaffold from frozen tissue on our dentin slice model. Additionally, our data sheds light on the importance of intact perivasculature in the potency of stem cells and regenerative capability of minced pulp. Our results showed confirming data that perivascular niche can be maintained intact in the minced pulp tissue, which can possibly explain the extensive cell proliferation potential of minced pulp as a source of mesenchymal stem cells. Overall, the result from our study highlights the scope of minced pulp potency and its cryopreservation in future endodontic regenerative procedures.

The thesis of Dhruvi Pravinchandra Patel is approved.

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2022

Acknowledgment

I would like to express my deep and sincere gratitude to Dr. Mo. Kang. His mentorship and immense support have been instrumental in my academic career. His guidance has played a significant role in my achievements.

I would truly like to thank Dr. Wei Chen for his constant support and vital inputs in the development of my postgraduate research project.

I would also like to thank my committee members Dr. Insoon Chang, Dr. Nadia Chugal and Dr. Reuben Kim for sharing their expert feedback.

I would like to thank my lab members, co-residents and dear friends for their support and uplifting spirit - Abdullah Alshaikh, Chaehwan Lee, Moein Sadrkhani, Gautam Shirodkar, Neha Batra, Laurel Martinez, Thomas Lin, and David Karwacki. I will forever cherish our camaraderie along the journey.

I would like to take this opportunity to acknowledge my parents Pravin, and Jagruti for being my backbone for the past five years, without their support my American dream would not have come true. I would like to thank my sister Rima who never stopped believing in me, and my two lucky charms – Adheesh and Avyaan. Last but not the least, I would like to thank my boyfriend Jeff for his invaluable support and encouragement through these years.

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Introduction

Regenerative endodontics is a biologically based technique designed to physiologically replace damaged tooth structures, including dentin and root structures, as well as the pulp-dentin complex. (Murray et al. 2017). It was first described by Nygaard- Östby in 1961 as a procedure for immature necrotic permanent teeth to achieve root end closure, with the additional goal of obtaining continued development of the root and thickening of the canal wall. Dr. Östby proposed the importance of blood clot within the root canal space and suggested that if the apical segment of the root canal is left empty it can potentially lead to ingrowth of cell rich connective tissue. As contrast to the conventional endodontic therapy where the root canal system is sealed and obturated with biocompatible foreign materials such as gutta percha and sealer, the regenerative endodontics procedure includes filling the canals with host's own vital tissue i.e., blood (Saoud et al. 2016). Traditional approaches of calcium hydroxide apexification and apical barrier techniques with mineral trioxide aggregate (MTA) have been used in the treatment of immature teeth with pulp necrosis, though generally there is no further root development, so the roots remain thin and fragile with a higher risk of fracture and tooth loss. (Trope et al. 2010). Terms like regenerative endodontics and apexification have been used interchangeably for several years. However, these two procedures have distinct and specific aims. Endodontic regeneration aims to achieve true pulp-dentin complex regeneration and at the same time provide increased dentin thickness and root end closer. On the other hand, apexification aims towards the formation to an apical barrier and gain further root length development, this procedure tends to leave the tooth inherently weak and susceptible to fracture.

A review of literature by Namour et al. 2014 showed several studies which have recognized pulp revascularization as a complex process which is based on the availability and the ability of the residual pulp, apical and periodontal stem cells to differentiate (Kahler et al. 2010; Torabinejad et al. 2011). It aims to revitalize the necrotic teeth and thereby giving it a chance to achieve further root length development as well as increased strength and thickness of the dentinal walls.

The aim of retaining the vitality is to maintain the defense mechanism in case of pathogenic stimuli. Contrary to the goal of regenerative endodontics which is to develop pulp-dentin complex like tissue, the histologic examination of the tissue formed after revascularization is more like fibrous connective tissue with often hard bone or cementum like calcifications in the root canal space (Becerra et al. 2013). Revascularization associated calcifications has been a challenge towards the goal of true endodontic regeneration. It has been shown that though the revascularization procedure is highly successful in resolving apical periodontitis and treatment of immature teeth with root canal infections, in 79% of the cases it leads to intracanal calcification and canal obliteration (Song 2017). Interrelation of hard dentin and soft pulp is important anatomically and structurally in regeneration of pulp- dentin complex. Proteins in dentin play a role in stimulating the signaling molecules to achieve the desired biological regeneration (Galler et al. 2020).

The goal of regenerative endodontics is to replace the necrotic pulp with scaffolds, healing promoting factors and cell therapies combined to achieve formation of a new pulp dentin complex (Galler et al. 2011). Adult mesenchymal stem cells are needed for pulp-dentin regeneration. Cell based approached have gained popularity in combination with tissue-engineering techniques. dental pulp derived stem cells (DPSC) were the first dental stem cells and studies have confirmed their odontogenic (Dissanayaka et al. 2015); neurogenic (Kuang et al. 2016); and angiogenic properties (Wang et al. 2013). Several studies have shown formation of pulp like tissue by using post-natal pulpal MSCs which includes DPSCs (Prescott et al. 2008) and stem cells from human exfoliated deciduous teeth (SHED) (Cordeiro et al. 2008). It has been established that unfractionated pulp tissue transplantation is essential for regeneration of entire pulp as well as the pulp-dentin complex (Murakami et al 2013) (Nakashima et al. 2011). Absence of the stem cell transplantation results in failure of pulp-dentin regeneration. To address the technical difficulties of cell-based approach in regenerative endodontics and its translation to a chair-side procedure, our lab presented a novel method for MSC transplantation into the root

canal system. Minced pulp tissue was used as the potential source of mesenchymal stem cells (MSCs), and explantation of these cells showed multipotential differentiation potential.

Mesenchymal stem cells are “captured” by the developing blood vessels and becomes pericytes. It is hypothesized that these pericytes retain their nascent stem cell potential and are reactivated as MSCs (Bautch et al 2008). Cell based regenerative approach is dependent on availability of intact and potent stem cells, maintenance of the perivasculature and intact pericytes can yield viable stem cells. Our approach of minced pulp tissue as a source of MSCs is scientifically significant as it eliminates the enzymatic processing of the tissue as well as collagenase induced changes in the gene expression. Mechanical fragmentation of adipose tissue was able to preserve the perivascular niche, whereas the enzymatically digested adipose tissue negatively influenced the perivascular content which also negatively influenced the influence of growth factors and cytokine secretion. (Vezzani et al 2018).

After showing the minced pulp as a source of MSC, we further wanted to evaluate the stem cell characteristics of minced pulp tissue under freezing conditions. The process of cryopreservation necessitates harvesting the donor stem cells by addition of cryo-preservatives. Maintaining the viability if the frozen unit is critical after thawing. Cryopreservation of hematopoietic stem cells has been rapidly expanding and the field of stem cell transplantation in the treatment of malignancies is booming. However, freezing of the tissue and stem cells is a challenging and sensitive process as the freezing process can induce injury by exposure to low temperatures. The two commonly described obstacles during cryopreservation are the “chilling injury” and “cold shock” which can render the tissue or cells to senescence (Fahy et al. 1995). The physiochemical reactions during the cooling and thawing processes can affect the survival rate of the frozen unit. This includes rupturing of the intracellular concentrates and disturbances in the plasma membrane.

Various Factors such is the freezing mediums, freezing temperatures, freezing rate, durability, thawing temperatures and conditions play a significant role in the outcome of

cryopreservation. Cryopreservation can be characterized based on the freezing conditions such as slow freezing, subzero nonfreezing storage, preservation in dry state. (Jang et al. 2017) Cryo-preservatives are crucial additives during the process of freezing as they prevent the formation of extra and intracellular crystals. Some commonly used cryopreservatives are ethylene glycol, glycerol, dimethyl sulfoxide (DMSO), propylene glycol etc. The mechanism of action of DMSO is by is potential to reduce the electrolyte concentration of the cells at any given temperatures. Several studies have shown use of 10% DMSO and 90% FBS for freezing of MSCs from umbilical cord blood. (Qui et al. 2012). Optimized cryopreservation of human dental pulp derived stem cells from enzymatically digested tissue for 24 hours has been shown. The results from this study showed that 10 % DMSO mixed 90% FBS in 1.5ml solution, tested to be ideal cryoprotectant in comparison to ethylene glycol and propylene glycol. (Woods et al 2009).

Other additives such as animal derived serums are essential as a source of nutrition to the cells during the freezing cycle. Apart from being a nutritive source it also helps in stabilizing the cell membrane, adapting the osmotic pressure, and protecting the cells from the free oxygen radicals formed during cryopreservation and long-term storage. (Curtis et al 2015). Keeping in mind the cytotoxicity and protective roles of DMSO as well the nutritive role of FBS our study tested varying concentrations of both in the freezing mediums. Different concentrations - 2%, 3% and 5% DMSO and propylene glycol has been tested and it was shown that 2% and 3% DMSO has negative protective role on human tooth germ stem cells.

In the present study we hypothesize that under ideal freezing conditions cryopreserved minced pulp tissue can retain its cell viability and mesenchymal stem cell potential. The main objectives of this study were to show that under ideal freezing conditions minced pulp derived mesenchymal stem cells (MP-MSC) can maintain their differentiation potential. By showing the presence of intact perivasculature in the minced pulp tissue we further open the scope of the extended cell proliferation potential of MP-MSC.

Materials and Methods

2.1 Sample collection and freezing variable:

Samples of freshly extracted non carious intact mature human teeth from patients between the age of 16-22 years were collected from the UCLA School of Dentistry, Oral Surgery Clinic. Immediately after extraction the teeth were stored in sterile Falcon tubes (corning) containing the 20 ml solution of α-MEM (Invitrogen, Carlsbad, CA) supplemented with 3% Antibiotic-Antimycotic (Life Technologies, CA). Sterile tubes containing extracted teeth were stored in a Styrofoam box with ice until they were transferred to the biosafety cabinet for dental pulp extraction and isolation. Dental pulp tissue from the extracted teeth was isolated within 12 hours of extraction.

Upon transfer to the biosafety cabinet, the teeth were washed three times with cold phosphate buffered solution (PBS), and all surfaced of the tooth were cleaned. Any attached periodontal and gingival tissue was removed with the help of sterile forceps and scalpel. The teeth were inspected for caries, fractures or any significant resorptive defects. Any teeth with open apices and exposed pulp tissues were discarded. A sterile metal wire cutter was used to crack the tooth at the cemento-enamel junction level. Once the tooth was sectioned, the coronal and the radicular segments were separated and the pulp tissue from the chamber and roots was extirpated using sterile micro tweezers. The pulp tissue was washed with PBS and placed in to a 60 mm petri dish containing primary culture medium comprised of α-MEM (Invitrogen, Carlsbad, CA) supplemented with 15 mg/mL gentamicin sulfate (Gemini Bio-Products, West Sacramento, CA), 20 mmol/L L-glutamine (Invitrogen) and 20% FBS (fetal bovine serum) (Invitrogen). Using sterile micro-scissors, the pulp tissue was minced into fine pieces (<1 mm^3). Four different freezing mediums were prepared with varying concentrations of primary media, FBS and DMSO.

For freezing medium 1 and 2 primary media was used and for freezing medium 3 and 4 FBS was used. 5% and 10 % of DMSO concentrations were selected as cryoprotectants. Following solutions of freezing variables were prepared:

a) Freezing medium 1 (F1) - 90% primary media + 10% DMSO

b) Freezing medium 2 (F2) - 95% primary media + 5% DMSO

c) Freezing medium 3 (F3) - 90% FBS + 10% DMSO

d) Freezing medium 4 (F4) - 95% FBS + 5% DMSO

Fresh unfrozen tissue was used as positive control and 100% FBS solution without DMSO was used as negative control. The minced pulp tissue from each sample was subsequently divided equally into 6 groups: 1 control, 4 freezing variable and 1 negative control.

2.2 Freezing of the minced pulp tissue:

1.5ml of each freezing medium was labeled on cryovials and the minced tissue was transferred to cryovials and 1.5ml of FBS was used for negative control group. The tissue fragments were exposed to controlled cooling at approximately -1 degrees Celsius/minute using a dump freeze method consisting of cryovials in an isopropanol bath in -80 degrees Celsius for 1 week following by plunge into liquid nitrogen for storage at -196 degrees Celsius for a period of 1 month.

After 1 month, cryovials were removed from liquid nitrogen and immediately thawed in 37°C water baths. Vials were exposed to slight agitation in the water bath to ensure complete melting of the ice. The tissue was then thoroughly washed with PBS for 10 minutes and transferred to 5 ml of basal medium containing α-MEM with 10% FBS and 5 µg/mL gentamicin sulfate. This was followed by centrifugation to separate any of the remanent cryoprotectants prior to tissue culture. Post centrifugation minced tissue was soaked in primary medium for 20 minutes.

2.3 Tissue culture and Cell expansion:

The control unfrozen tissue was transferred to a 48 well plate with fresh primary culture medium and immediately incubated in 80% humidity, 5% carbon dioxide atmosphere at 37 degrees Celsius on the same day of sample collection (Liang et al. 2018). Similarly, post thawing the frozen groups were transferred to 48 well plate containing fresh primary culture medium and incubated in 80% humidity, 5% carbon dioxide atmosphere at 37 degrees Celsius. Control and frozen samples were cultured for 14 days and serially passaged after 80% confluency. MP-MSCs were maintained in basal medium consisting of α-MEM (Invitrogen, Carlsbad, CA) with 10% FBS (Invitrogen) and 5 μg/mL gentamicin sulfate (Gemini Bio- Products, West Sacramento, CA).

High magnification images at were obtained under the microscope and photographic documentation for done on days 3,5,7,12 and 14. Area of cell expansion for each group was measured. Cell replication kinetics were determined based on the total number of cells collected after 14 days and cell account analysis was performed as described in our previous studies (Liang et al. 2018). Cell count and area of cell expansion was quantified and plotted.

2.4 Reverse transcription Polymerase chain reaction to show expression of osteo/odontogenic markers

Tissues from control and frozen group were cultured till they reached 70-80% confluency. Total RNA was isolated from the cells using TRIzol reagents (Invitrogen) and repetitive mixing. Chloroform was added to the homogenized sample after incubation for 5 minutes. Interphase aqueous solution was obtained after centrifugation for 15 minutes and the upper aqueous layer was preserved for RNA precipitation. The organic phase was saved for DNA isolation. Isopropanol was added and after 10 minutes of centrifugation RNA precipitate was obtained. RNA wash was done with 800μL of ethanol and the quality and measure of total RNA was assessed using NanoDrop Spectrophotometer (ThermoFisher Scientific).

Amplification of cDNA was completed with SYBR Green I Master Mix (Roche) with the help of Light Cycler 480 II real-time PCR system (Roche), following manufacturer's instructions. All experiments were triplicated. The thermal settings used for initiation of heat denaturation were 95 degrees Celsius for 10 minutes, followed by 45 cycles of 95 degrees Celsius for 10 seconds and lastly by 58 degrees Celsius for 45 seconds and 72 degrees Celsius for 10 seconds to complete denaturation. GAPDH was used as an internal control. Determination of the second derivative of the Cq value was done by comparing the gene of interest to GAPDH for fold expression differences of amplification following the manufacturer's instructions (Roche).

2.5 Odontogenic differentiation assay

MP-MSCs derived from the control and frozen groups were cultured and seeded on 6 well plate. Odontogenic induction media consisting of basal medium conditioned with 100 μmol/L L-ascorbic acid 2-phosphate (Sigma, St Louis, MO), 9mmol/L KH_2PO_4, 10 mmol/L β-glycerophosphate, and 9.8 nmol/L dexamethasone (Sigma, St Louis, MO) was then added to the cell culture and the control group for this experiment were cultured in basal medium alone. The medium was changed every 48 hours.

Alkaline phosphatase staining kit (Sigma) was used to stain the cells on days 7 and 14 after induction. Dulbecco's phosphate buffered saline (DPBS) was used to wash the cells prior to fixing with acetone/citrate 1:2.5 for 30 seconds. After fixation cells were washed again with distilled water for 45 seconds and added to alkaline-dye mixture for 30 seconds. Solution A was prepared by mixing 1 mL of Fast Violet B Salt with 1 mL of sodium nitrite solution for 2 minutes. Solution B was prepared by 1 mL of Naphthol AS-MX phosphatase alkaline solution in 48 mL of distilled water. The alkaline dye mixture was prepared by mixing solutions A and B. 2 mL of the mixture was added to each well for 30 secs. The cells were incubated for 10 minutes in the dark. The samples for control and frozen group were seeded in triplicates. Images were captured under the microscope.

Equivalent number of cells were seeded in a 6-well plate for Alizarin Red Staining and were also triplicated. Control of each group were cultured and maintained in basal medium. Cells were exposed to the calcifying conditions of odontogenic induction medium for 14 days and medium was changed every 2 days. On day 14, cells were rinsed with DPBS and fixed using 70% ethanol. 40mM Alizarin S Red with pH 4.2 was used to stain the cells at room temperature for 10 minutes with gentle shaking. All the unbound Alizarin S Red was removed by sterile water rinse. Staining was then quantified by de-staining with 10% acetyl pyridinium chloride (Sigma) and the dye absorbance was measured at 405 nm optical density on the multiplate spectrometer. Staining was observed under the microscope and photographs were taken.

2.6 Dentin Slice/Scaffold model

Freshly collected molars and premolars were transversely sectioned at the cervical level using diamond bur in presence of copious cooling with sterile saline. Dentin slices of approximately 1mm thickness were obtained. Immediately after cutting they were placed in sterile Falcon tubes containing 70% alcohol and were autoclaved.

The central pulp cavity space was then filled with sodium chloride (Sigma), followed by poly-L-lactic acid (PLLA) (Goodfellows, US) which was dissolved in chloroform solution. This PLLA was poured in the chamber cavity it was left open in the fume hood overnight for adequate evaporation of chloroform. The polymerized PLLA dentin slices were then washed with distilled water. Minced pulp tissue from control and frozen groups were seeded in the center of the scaffold and incubated for 1 hour. The dentin slice with scaffold and embedded tissue was then transferred to 6 well plate with fresh primary culture medium and was cultured for up to 21 days. Post culture the dentin slices were fixed in 10% formalin at 4 degrees Celsius for 24 hours. Samples were then sent for paraffin mounting and sectioning to translational pathology core laboratory (UCLA) and hematoxylin-eosin staining was performed.

2.7 Immunofluorescence staining

Samples of minced pulp tissue from control and frozen groups prior to tissue culture were collected and fixed using 4% paraformaldehyde in 4 degrees Celsius for 24 hours. For comparison between the fragmented and unfragmented pulp tissue, whole pulp tissue was extirpated from a maxillary premolar tooth and processed similarly for the immunofluorescence staining. The samples were then mounted on paraffin blocks and sectioned into 5 µm. Slides with mounted sections was placed in the oven for 60 minutes. The sections were then rehydrated by xylene and ethanol series and final washing with running water. Unmasking solution was prepared by mixing of 500 ml distilled H2O with 5.46 ml of antigen unmasking solution. The unmasking solution was boiled for 7-9 minutes, and the slides were immersed in the solution for 25 minutes at 10% microwave power. The samples were then cooled on ice for 20 minutes at room temperature, and washed with 1x phosphate buffered saline, 0.1 Tween (PBST) for 5 minutes. Pap pen was used to mark around the tissue sections on the slide. For permeabilization 25 µL of Triton 100X (Sigma-Aldrich, Germany) mixed with 5 ml of PBS is freshly prepared, drops of this solution were added on the slides and left for 60 minutes. Samples were washed again with PBST for 5 minutes. Solution containing 900 µL methanol and 100 µL hydrogen peroxide was then added to the slides and left for 15 minutes followed by another PBST wash for 5 minutes. Blocking buffer was prepared using 5% bovine serum albumin (BSA) in PBST and added to the slides for 1 hour. This solution helps to block the non-specific binding. Mouse antihuman NG2 (BD Biosciences), and rabbit anti-human PDGFRβ (Abcam, Cambridge, UK) diluted in the antibody diluent (Life technologies, Carlsbad, CA) were added and incubated at 4 degrees Celsius overnight. Primary antibody was then added left overnight. PBST wash for 5 minutes was done followed by addition of species- specific secondary antibody. Following fluorochrome-conjugated secondary antibodies were used: anti-mouse-Alexa 555 IgG, anti-rabbit-Alexa 647 IgG and conjugated 488 (Life technologies). Sections were incubated for 1 hour at room temperature. 1 drop of DAPI (Life technologies) was placed on each sample for 3

minutes in absence of direct light to stain the nuclei. Immunohistology mount liquid (Southern Biotech) was added on the samples. Images were captured at 60x, 120x magnification using a florescence microscope and processed using FluoView™ laser scanning confocal microscopy (Olympus Life Sciences).

Results

3.1 Evaluation of ideal freezing medium:

Thirty-two freshly extracted teeth were collected from UCLA Oral and Maxillofacial Surgery clinic between July 2018- December 2019. Culturing of the unfrozen minced pulp tissue showed similar results as our previous study with high cell proliferation and expansion rates. Minced tissue from each sample was divided into 6 group, samples for control was cultured immediately on the day of extraction (Figure 1). For the frozen group the four variables were freezed under similar rates and conditions for a period of 1 month. One-month later these frozen group tissues were cultured under identical conditions and photographic documentation was done on days 3, 5, 7 and 14. Based on cell expansion rate and cell count, freezing mediums with 10% DMSO shows higher numbers of cells migrating from the frozen tissue as compared to the samples with 5% DMOS suggesting the importance of the cryoprotectant (Figure 2a). The possibility of the crystallization of the intra and extracellular components in the lower DMSO group explains why the cell migration was delayed and lower. There was significant reduction in the cell count of the frozen tissue when compared to the control group, which is an expected phenomenon due to the freezing cycle injury. However, to support our hypothesis we noted that the cells derived from frozen group with 10% DMSO were still viable as they showed expansion to 80% confluency though it was at a slower rate as compared to the control group. On the other hand, the negative control group with no DMSO did not yield any cells suggesting tissue disruption and cell death during freezing. These results concluded that the freezing medium containing 10% DMOS with 90% primary medium was ideal and maintained the highest number

of viable cells after 1 month of freezing time. Based on this outcome we selected the two groups with 10% DMSO for following experiments.

3.2 Morphological characteristics of MP-MSCs after freezing:

MP-MSCs derived from frozen group were evaluated under 10x magnification. Microscopic examination showed that the cells migrating from the frozen tissue was identical to the MP-MSCs from the unfrozen group resembling the spindle shape that is characteristic of mesenchymal cells. Cells from the experimental frozen group showed similar pattern of proliferation and expansion for up to 14 days and further tissue passage (Figure 2b).

3.3 mRNA expression patterns of genes associated with odontogenic differentiation:

After osteogenic induction for 7 days, mRNA expression of various osteogenic and odontogenic genes was evaluated. OCN (Osteocalcin) is a major bone protein. It plays a significant role in the regulation of the bone matrix mineralization and potentially controls the size and speed of crystal formation. (Riccio et al 2010). Another key osteogenic marker is RUNX2 which plays an important role in the signaling pathways involved in differentiation of osteoblasts. RUNX2 is highly expressed during early stages of osteogenic differentiation. (Deng et al. 2008; Granéli et al. 2014). DSP is the most prominent non-collagenous protein in dentin. It plays a significant role in dentin mineralization and has shown to increase the fracture resistance (White et al. 2007). Collagens 1 and 3 are the most abundantly present in dentin and pulp, synthesized and controlled predominantly by odontoblasts. They play a role in dentinogenesis by providing an organized scaffold. (Goldberg et al. 2012). RT-PCR results showed that after odontogenic induction for up to 7 days there was a significant increase in the mRNA expression of OCN, RUNX2, DSP, Collagen I and III in the control group (Figure 3). In the experimental frozen group, increasing expression of Collagen 3, OCN and Collagen 1 was noted on day 7, however RUNX2 did not show significant expression in the frozen group. Expression of these

markers suggests that MP-MSCs derived from frozen tissue are capable of maintaining their odontogenic differentiation potential.

3.4 ARS and ALP staining shows mineralization potential:

To further investigate the mineralization potential, we performed ARS and ALP staining post osteogenic induction for 7days. Alizarin Red Staining is to demonstrate the presence of calcium salts and is used to analyze the mineralization potential. Robust mineralization was expressed by cells from both the groups between days 7 to 14 with (Figure 4). Quantification of ARS absorbance showed no significant difference between the control and the frozen group suggesting that though the number of cells extracted from the frozen group were lower, however their differentiation potential remained unaltered (Figure 5a). ALP staining also showed increasing intensity on day 7 after culture in induction media which confirmed increased osteoblastic activity. (Figure 5b)

3.5 Dentin Slice model showing cell migration from the MP:

Histological examination was done using H&E staining showed that cells from the frozen minced pulp tissue in presence of the PLLA scaffold were able to migrate from the tissue explants towards the dentinal wall and were attached to be scaffold. Minced pulp tissue was embedded and showed adherence to the scaffold and remained an active source of viable cells. (Figure 6)

3.6 Immunofluorescence staining of minced and whole pulp tissue shows intact perivasculature:

Immunofluorescence staining was preformed to show the expression of pericyte markers such as NG2, PDGFRβ. Our results showed co-expression of these pericyte markers along with stem cells markers such as CD146 and STRO1. The expression of these markers suggests an intact perivasculature in the minced pulp tissue for both control and frozen group. Additionally,

we showed similar pattern of pericyte expression in the longitudinal section of intact pulp tissue. These findings provide promising results comparing the minced and intact pulp tissue. Pericytes are found around the vessels in the body of the tissue (Mills et al. 2013) and they have stem cell like properties and ability to differentiate into osteoblasts, chondrocytes. Immunofluorescence staining revealed expression of neural/glial antigen 2 (NG2) and platelet derived growth factor receptor beta (PDGFRβ) along the periphery of the blood vessels suggesting that the perivasculature remained intact in the minced pulp tissue. We also repeated the experiment for tissue which was cultured for up to 80% confluency. To support our proposal of MP as a potent source of MSCs we found that even though there were a smaller number of cells in the cultured tissue, the pericytes were still expressed along the endothelial lining suggesting that this tissue can still release viable stem cells. Stem cell markers CD146 was often noted to be co-expressing with the pericyte markers suggesting the latter's co-relation to MSCs. Thus, the immunofluorescence results confirmed that the perivascular niche is maintained intact in the minced pulp tissue.

Discussion

Cryopreservation has been established for several decades to permit long term storage of tissues and organs. However, this process comes with its challenges to maintain the subcellular constituents and the details of the cell ultrastructure during the period of freezing and recovery from subzero temperatures. The process of cryopreservation is a sensitive procedure and includes specifications of the freezing rate, conditions, cooling conditions as well as thawing and post thawing settings. With increasing clinical need for stem cells in the treatment of malignancies and other conditions like Parkinson's, the need for effective and clinically efficient methods for preserving these cells is needed. The potential of embryonic stem cells, to produce a wide ranged supply of normal, differentiated cells in the past decades has brought attention on the importance of these cells in tissue engineering as well as gene and cellular therapy. (Seong et al. 2010).

The progenitor stem cells derived from dental pulp can generate dentin-like hard structure comprising of odontoblasts-like cells which encompasses the pulp-like interstitial tissue. (Gronthos et al. 2000). However, historically all studies used enzymatically digested tissue derived DPSCs which required the tedious task of in vitro cell culturing. Numerous studies have investigated the possibility of pulp cells harvested from tissue explantation with a futuristic idea of eliminating troublesome task of DPSC isolation. It has been shown that explant derived human dental pulp stem cells expressed superior release of Ca^{2+} upon treatment with endothelin - 1 and there by showing higher proliferation and differential potential than conventionally derived DPSCs. The time of isolation method promotes an extended release of progenitor stem cells from the perivascular structures. (Spath et al. 2010). In line with these finding our lab proposed utilization of direct tissue grafting in regenerative endodontics. This method demonstrated that the tissue explants in the form of minced pulp tissue were capable of extended cell proliferation and differentiation potential. Recently harvesting of dental pulp tissue from sources such as extracted teeth and via pulpotomy achieved through coronal access has been shown. (Vendramini et al 2021).

The focus of our current investigation is to venture in the field of cryopreservation of the tissue explants. We were successfully able to demonstrate the cell proliferative potential of these fragmented tissues after a freezing period of 1 month. Early studies in this field showed a lower rate of 16% recovery post freezing using the standard slow vitrification method. (Reubinoff et al. 2001). Another study by (Zhou et al 2004) showed similar results for embryonic stem cells where the surviving cell colonies were reduced. The results from our current studies are in line with previous studies as the frozen experimental group did show viable cell colonies however, they were reduced in numbers. The significant difference, however, is that out study focuses on the freezing if the tissue fragments itself which serves as a clinically relevant and achievable goal, specifically for endodontic regeneration. We manipulated the concentrations of DMSO and FBS for our experimental group. Our results based on *in vitro* cell culture analysis showed that a

concentration of 10% DMSO with primary medium containing 20% FBS in αMEM with 3% antibiotics provided the highest number of surviving colonies. Our results are in line with (Lui et al 2008) who demonstrated that 10% DMSO does not pose any toxic effects on the cell viability and differentiation potential of adipose-derived stem cells.

Culture of the MP from the frozen group yielded viable cells for up to two passages. Morphologic characteristics of these MP-MSCs extracted after freezing remained the same and the cells showed spindle shaped fibroblast like appearance as well as showed exponentially proliferation. Mesenchymal stem cells derived from dental pulp tissue shows notable pluripotent mechanism. A strong multi-lineage potential was shown in clonal studies, where MSC differentiated to five mesenchymal lineages: bone, cartilage, fat, muscle, and fibrous tissue (Sarugaser et al. 2009). The clinical therapeutic use of MSCs is highly depending on this differentiation potential of which the osteogenic differentiation and mineralization potential are crucial for pulp-dentin complex regeneration. Our investigation shows that the MSCs even after undergoing the freezing cycle can maintain its osteogenic and odontogenic differentiation potential which was affirmed by molecular analysis results showing increasing gene expression patterns for OCN, DSP, DPP and Collagen 1 and 3. Quantification of the mineralization shown by ARS further confirmed that the freezing mechanisms did not alter the mineralizing capacity for the minced pulp tissue.

The major components essential for regenerative endodontics are stem cells, growth factors and a scaffold to hold the matrix together. As previously described these scaffolds should be biodegradable porous materials to enhance the cellular growth by providing a stable mechanical support as well as provide porous channels to resemble the extracellular matrix. The dentin slice model used in our previous study with PLLA scaffold was also used here and it is identical tooth root model used by (Huang et al. 2009). This model enables to replicate the actual clinical scenario, or the root canal system housed with the dentin and study the interaction between the MSCs with the dentinal proteins. Future experiments focusing more on the ex vivo

relationship and the signaling mechanism between the cells and dentin surface needed. Our results confirmed that after seeding of the frozen MP tissue the cells migrated from the tissue and moved towards the tissue periphery and inner dentinal wall. This interaction is beneficial to promote the ability of the MSCs to undergo odontogenic differentiation as shown in our previous study.

Furthermore, our study showed the intact perivasculature of the minced pulp tissue by expression of perivascular markers which explained the extended cell proliferation potential for the tissue explants. Stem and progenitor cells in the embryonic blood vessels reside in the nascent blood vessels and may contribute to the formation of endothelial vascular cells, pericytes and endothelium. The adult blood vessels provide a niche for stem cells and act as a reservoir of pericytes which may contribute to mesenchymal lineage and harbor the adventitial progenitor cells. Thereby maintaining this perivasculature can result in high yield of mesenchymal cells. These pericytes have a potential to retain their nascent stem cell property and they can differentiate into MSCs when the tissue is exposed to an insult. A recent study (Vezzani at al. 2018) showed that the micro fragmented adipose tissue is significantly enriched in pericytes. The secretome array showed higher protein secretions as well as higher concentration growth factors and cytokines corelated with tissue repair and regeneration in the micro fragmented group as compared to the enzymatically digested tissue. Immunofluorescence analysis of minced pulp tissue of both control and frozen group showed identical expression of pericyte markers NG2 and PDGFRβ along the periphery of the blood vessels. We also demonstrated co-expression of the MSC markers such as CD146 and STRO-1. Our results were in line with the findings from micro fragmented adipose tissue and thereby we determined that the fragmented and minced pulp tissue is a more potent source of MSCs than enzymatic digestion of the pulp tissue.

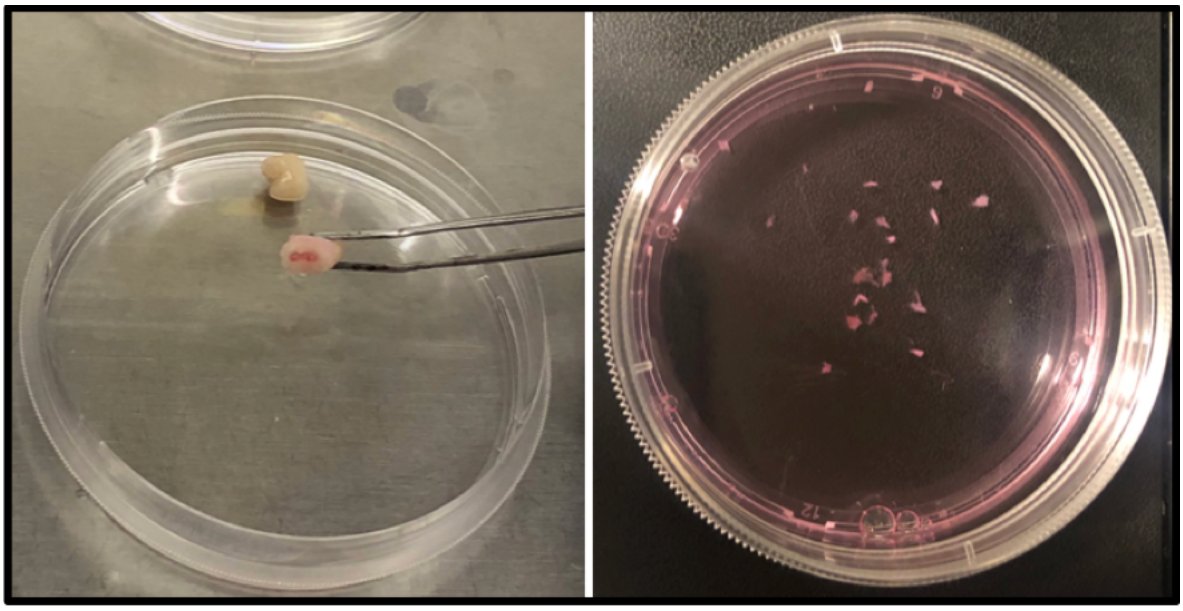
In conclusion, our study opened the scope of pulp tissue banking as a possible option for future regenerative endodontic procedures. Comparison of the frozen tissue with the unfrozen group sheds light on the resilience of the dental pulp tissue under freezing conditions. We

simultaneously strengthened our previous results promoting direct dental pulp grafting by showing the intact perivascular niche in the minced dental pulp tissue. We provide a fundamental rational towards harboring and cryopreserving the sterile pulp tissue from non-carious third molar extractions and implementing its use for future regenerative endodontic procedures.

Figures and Legends

Figure 1. Sample collection and four freezing test variables.

Isolation of minced pulp tissue and cryovials with the four experimental freezing medium groups.



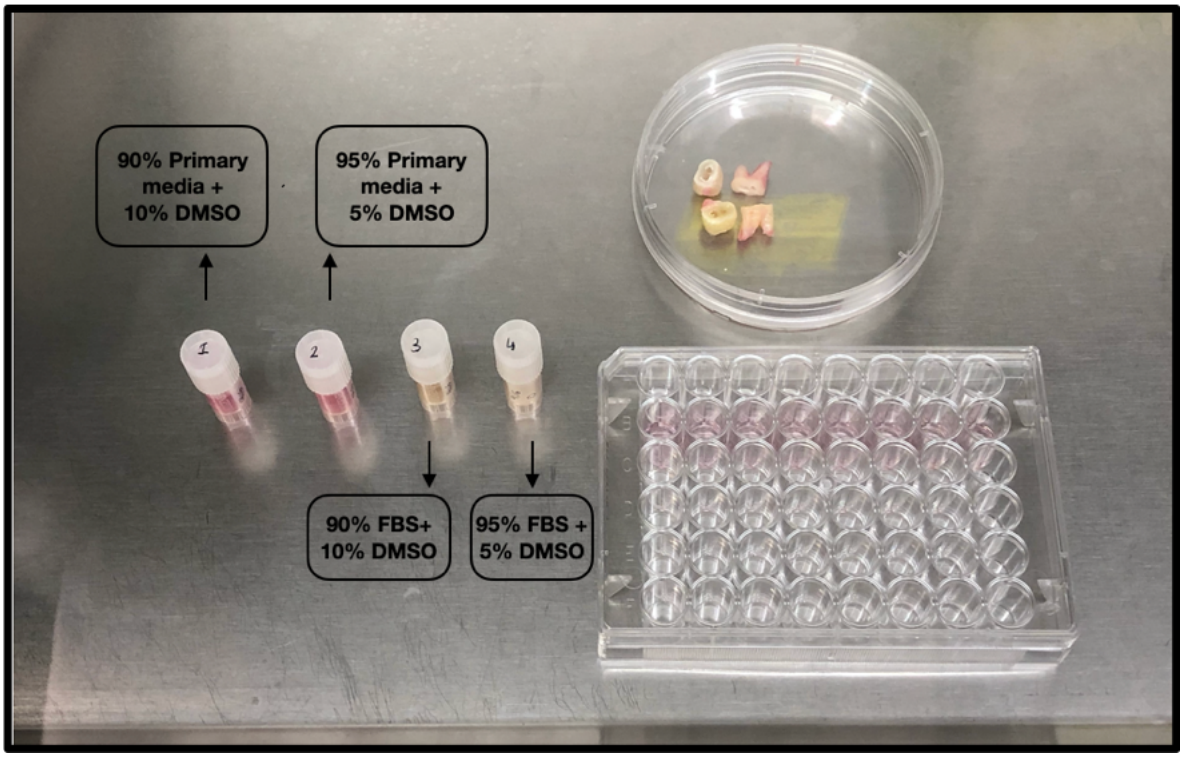


Figure 2. Comparative analysis of cell migration and proliferation.

a) Minced pulp tissue culture with fresh primary medium showed cell migration in the control group on as early as 7 days whereas for the frozen samples it was delayed. Viable cells migrating from frozen tissue was morphologically indistinguishable from the control unfrozen group. Images captures at 100X magnification under phase contrast microscopy.

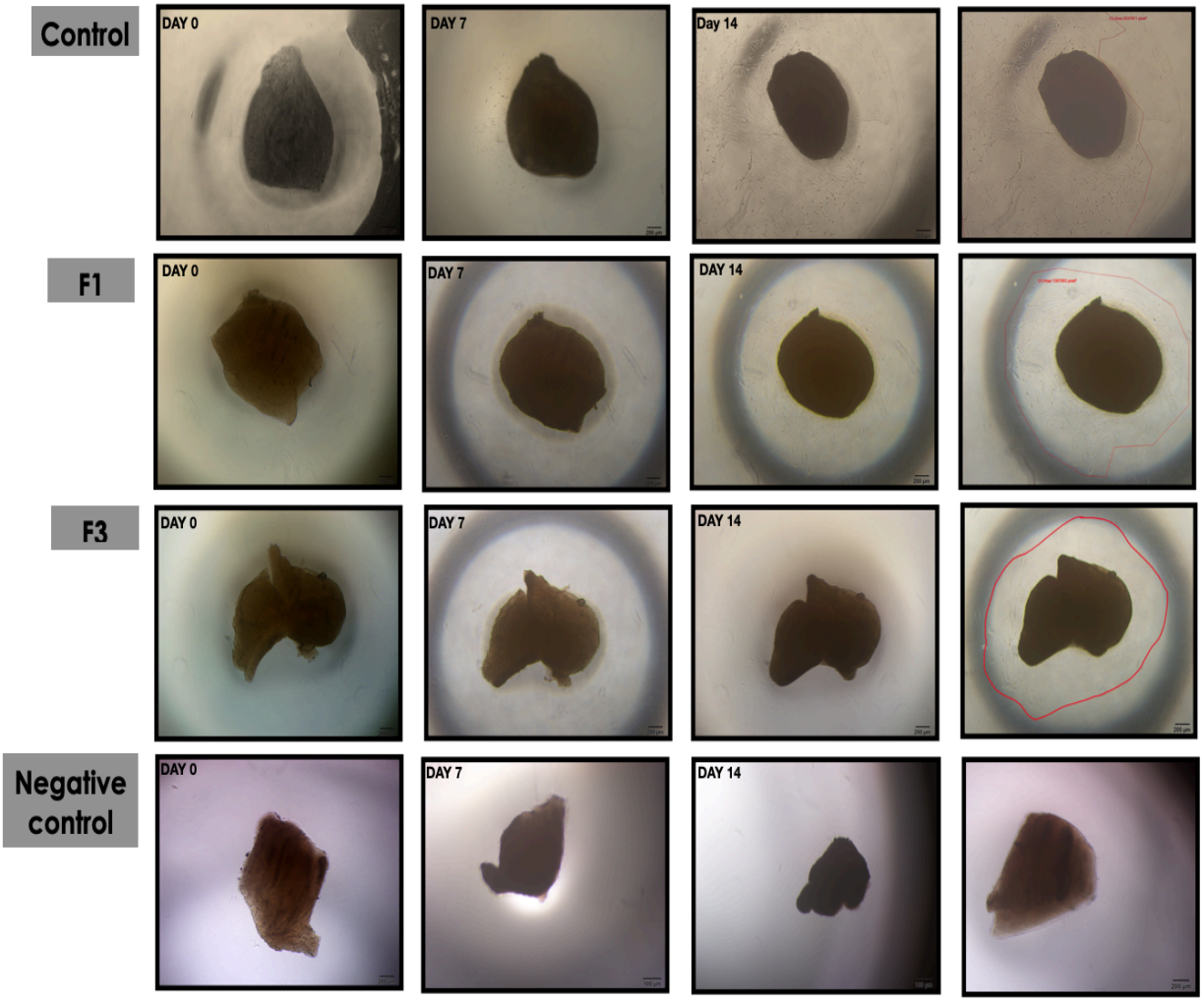
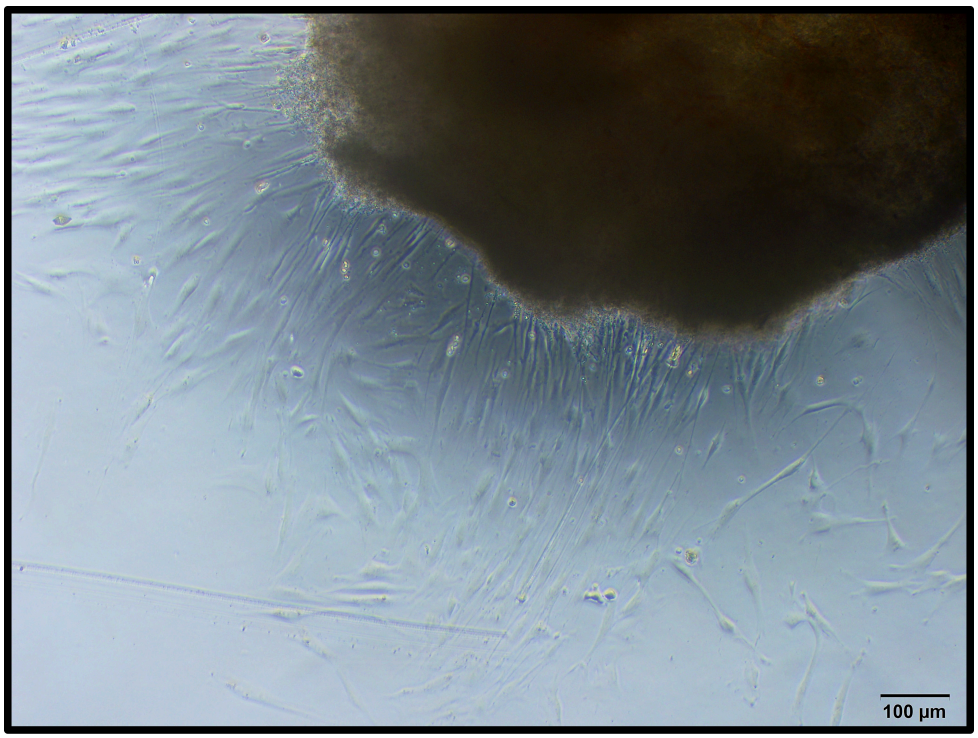


Figure 2. Comparative analysis of cell migration and proliferation.

b) High magnification microscopic images showing identical morphology.

CONTROL



FROZEN (F1)

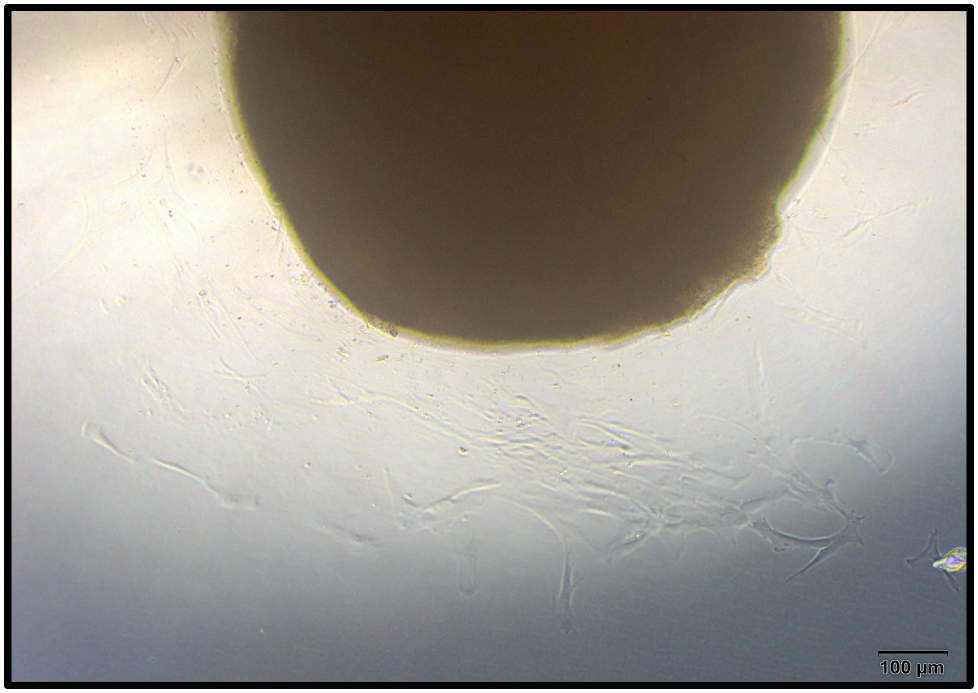
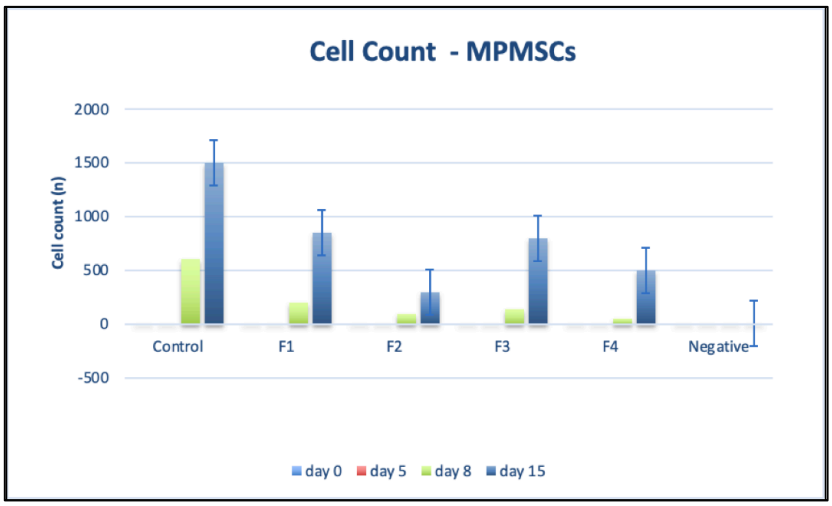


Figure 2. Cell count and area of cell expansion.

a) Significantly lower number of surviving colonies were seen in the two freezing groups with 5% DMSO.



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b) Frozen group shows reduced area of expansion and proliferation. Freezing medium containing 90% primary medium + 10% DMSO was determined to be ideal amongst the four.

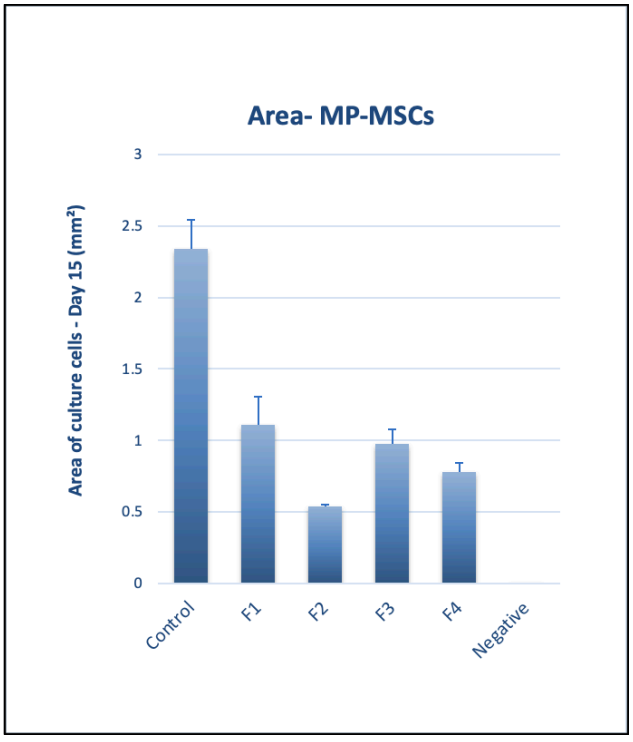
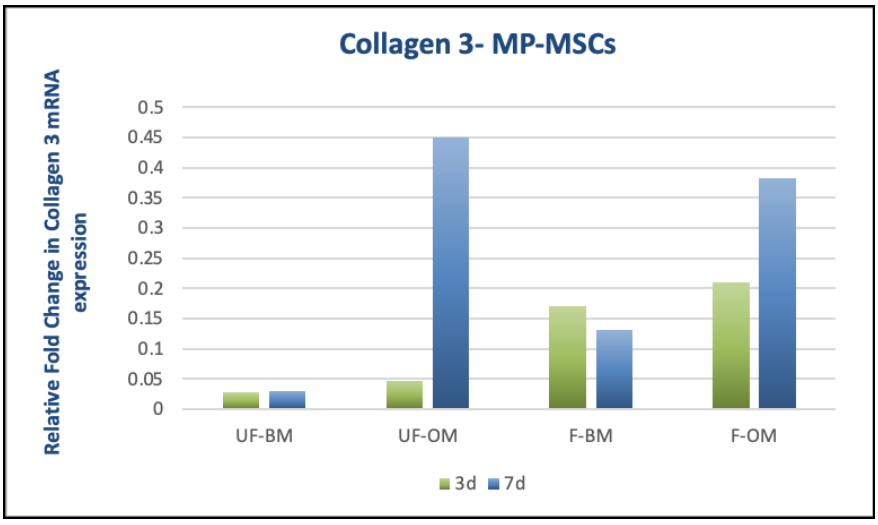
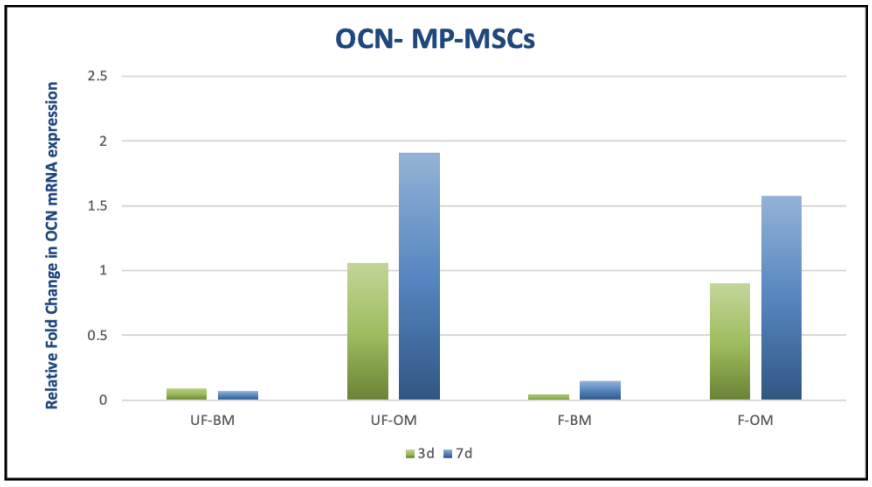
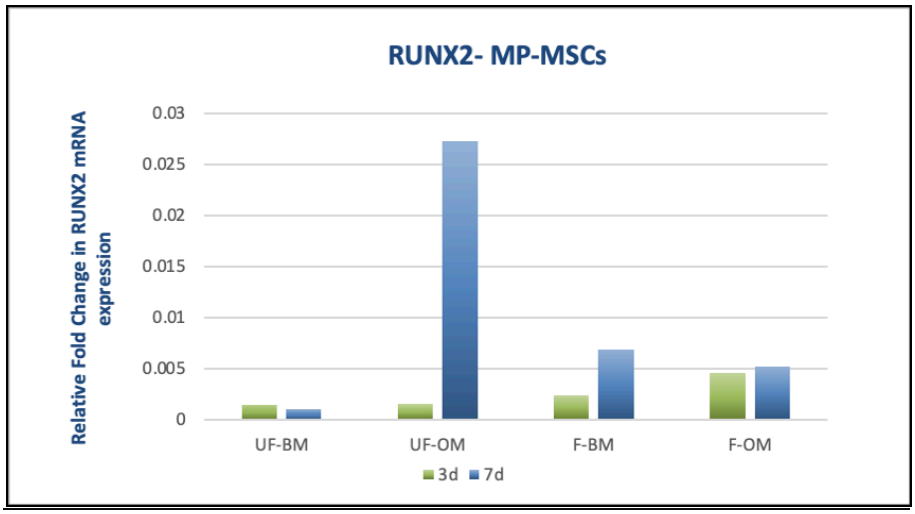


Figure 3. mRNA expression pattern of odontogenic and osteogenic markers.

mRNA expression of osteogenic and odontogenic markers. Expression of OCN, Collagen I and III showed no significant difference between the control and test groups.






RUNX2- MP-MSCs
Relative Fold Change in RUNX2 mRNA expression
0.03
0.025
0.02
0.015
0.01
0.005
0
UF-BM
UF-OM
F-BM
F-OM
3d
7d

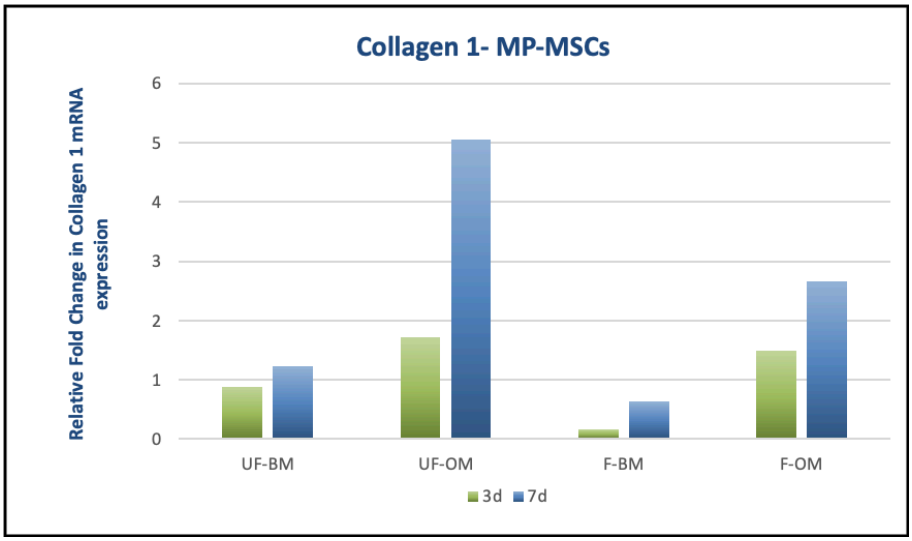

Collagen 1- MP-MSCs
Relative Fold Change in Collagen 1 mRNA expression
6
5
4
3
2
1
0
UF-BM
UF-OM
F-BM
F-OM
3d
7d

Figure 4. Mineralization shown by ARS and ALP staining.

a) ARS showed robust mineralization on day 14 for all groups under microscopic examination. No significant difference was seen between the unfrozen control and the frozen group samples with 10% DMSO.

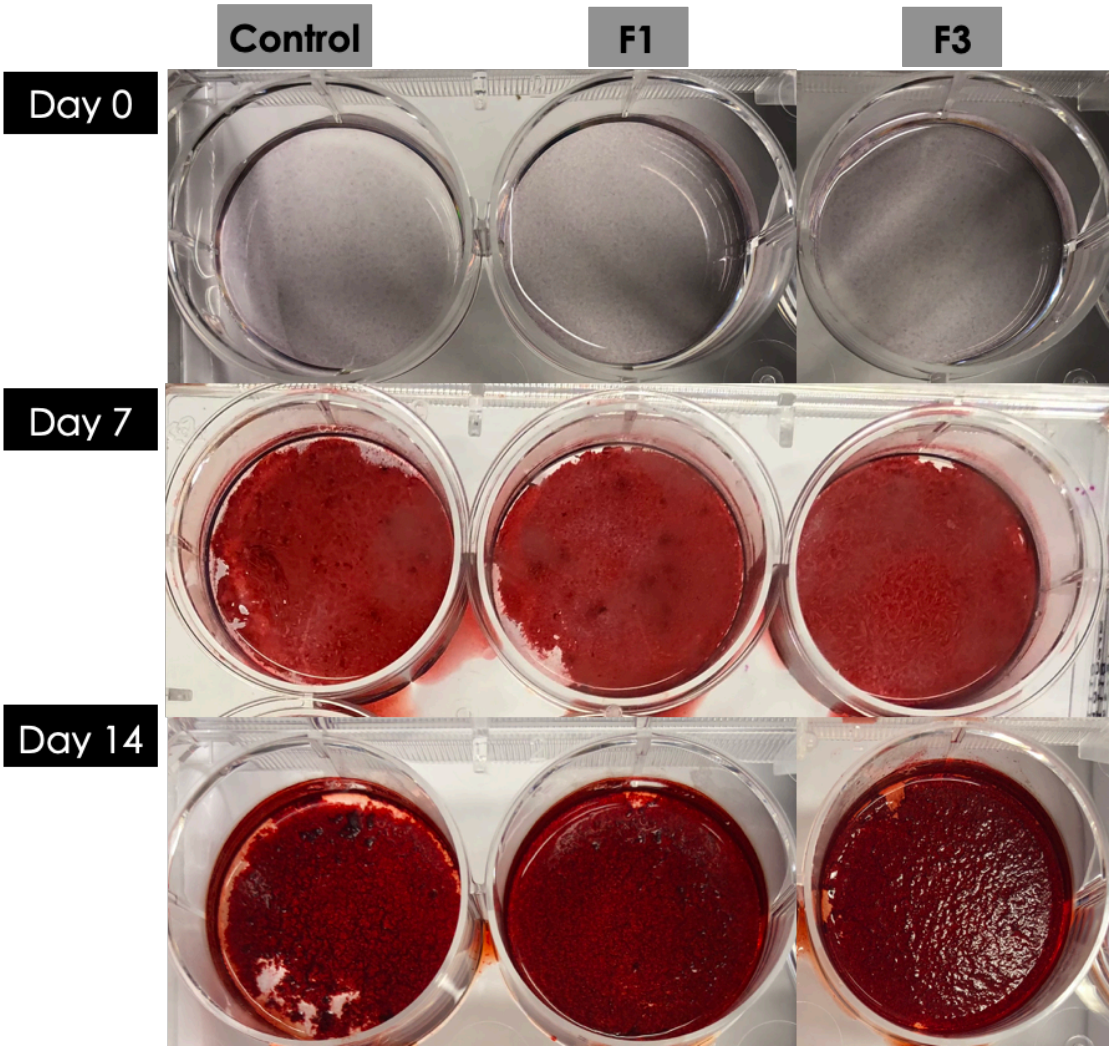
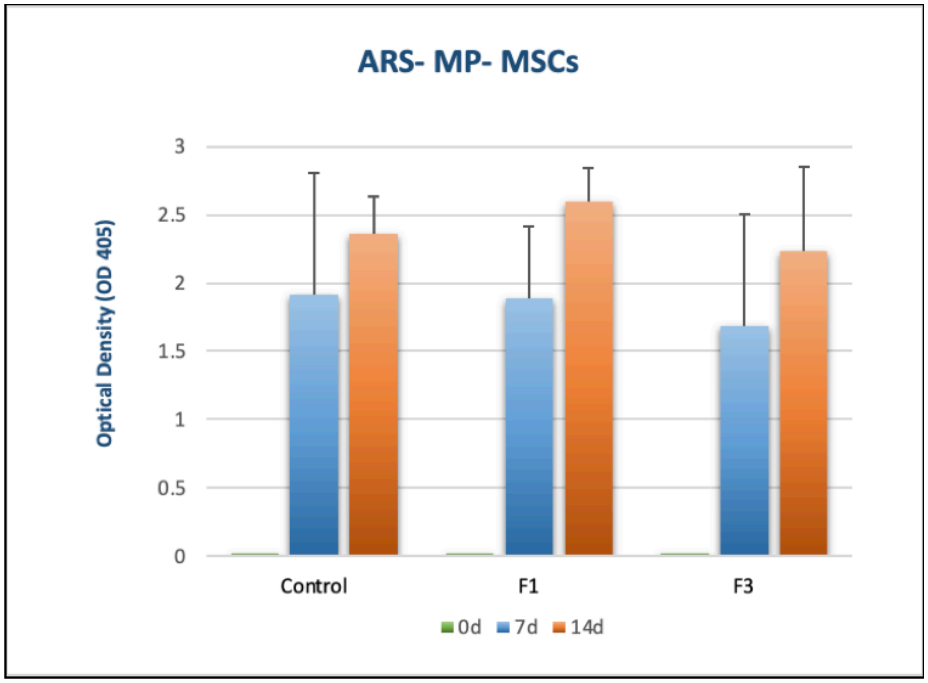


Figure 5. Mineralization shown by ARS and ALP staining.

a) Quantification of ARS absorbance showed no significant difference between the unfrozen control and the frozen group samples with 10% DMSO.

a)



b) ALP staining showed calcium nodules after induction.

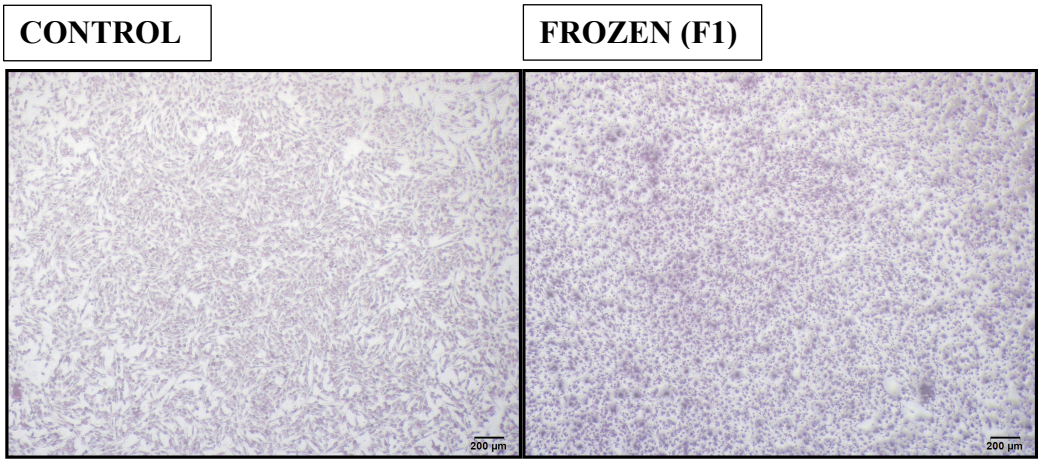


Figure 6. 3D Dentin Slice model with PLLA scaffold.

a) Microscopy showing minced tissue embedded in the porous PLLA mesh and migrating of cells from the tissue periphery. (arrows)

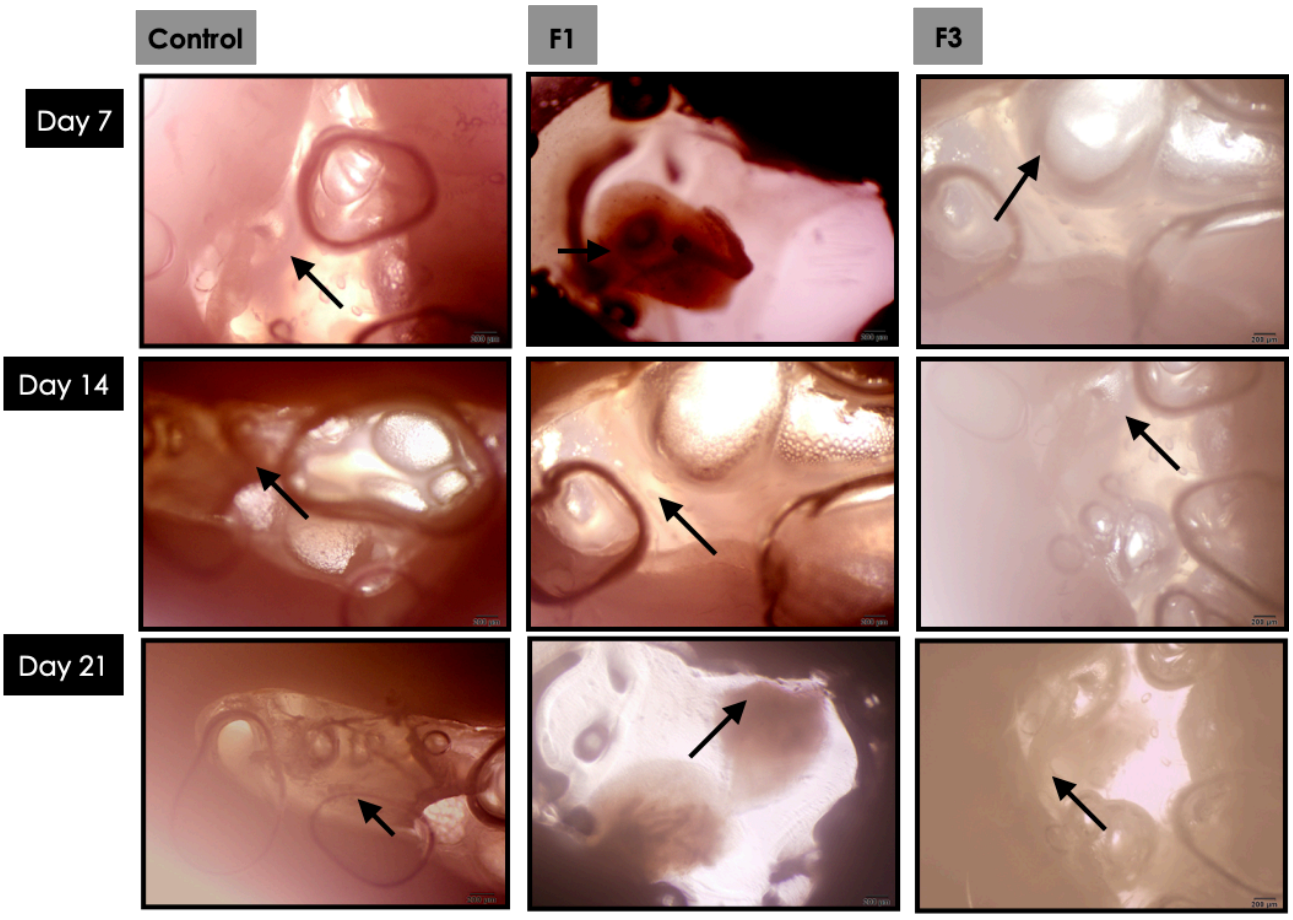


Figure 6. 3D Dentin Slice model with PLLA scaffold.

b) H&E staining showing cells adhering to the dentinal wall surface on the dentin slice.

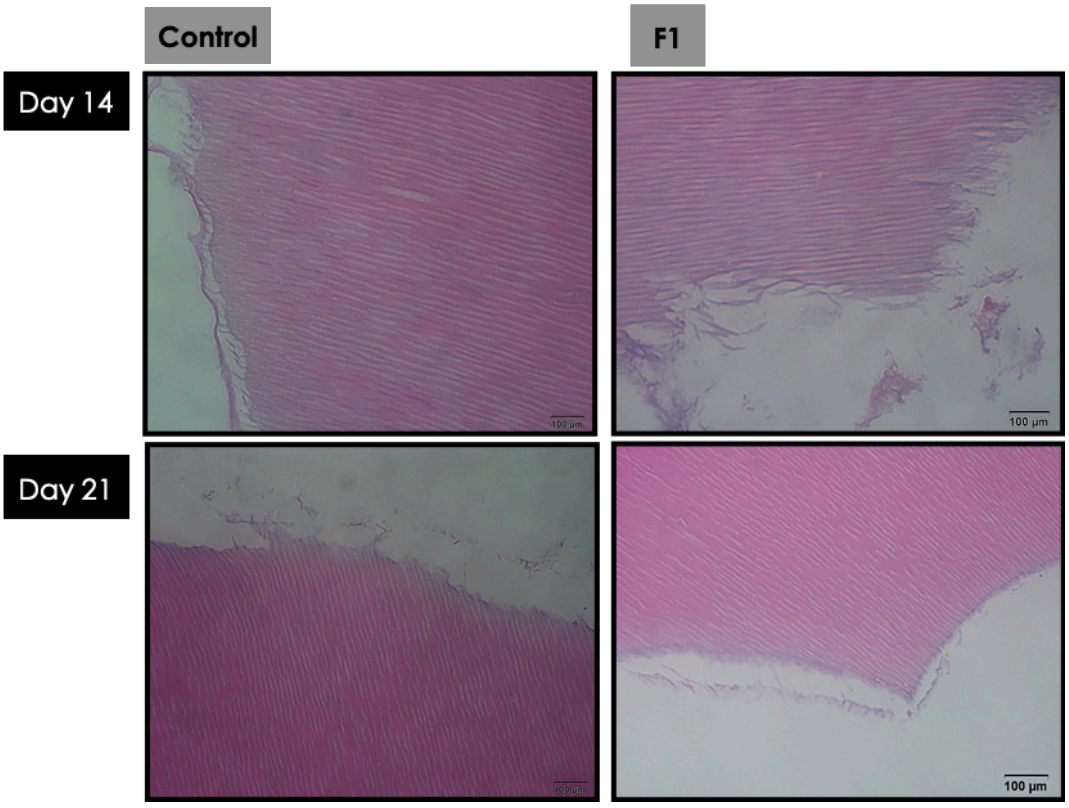
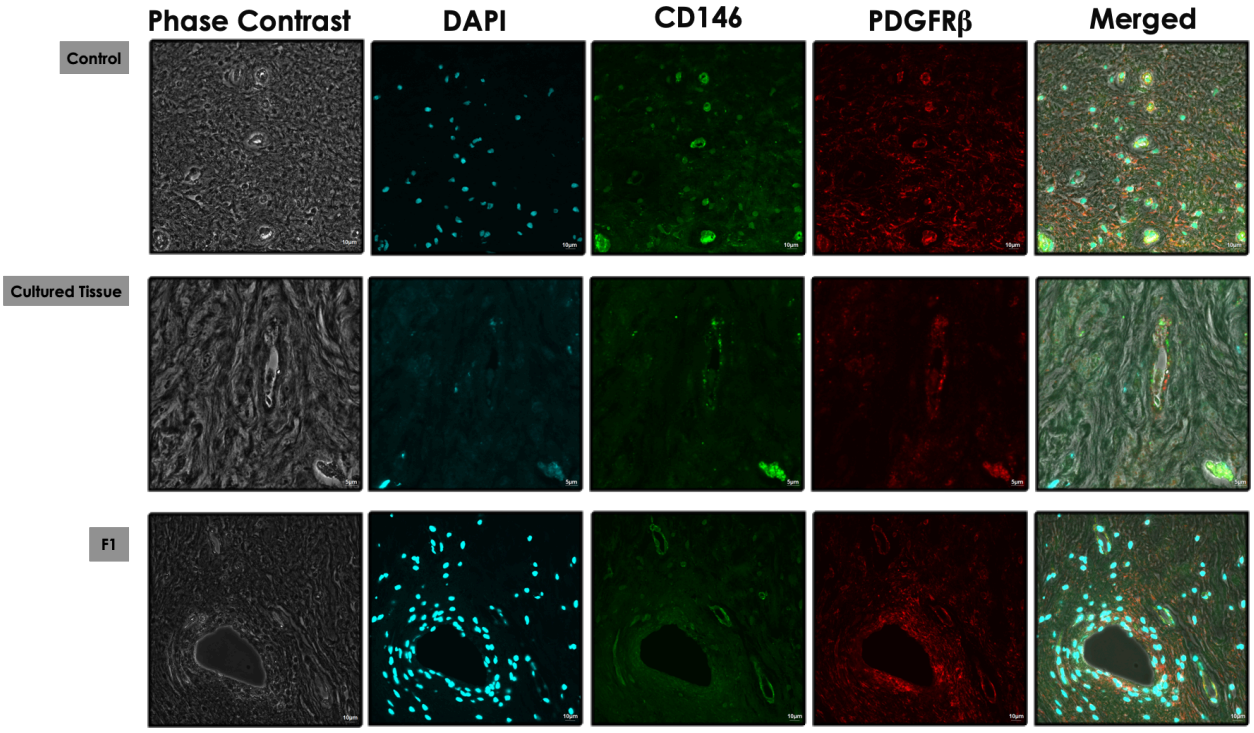


Figure 7. Immunofluorescence microscopy showing pericyte markers.

a) Expression of pericyte marker PDGFRβ and MSC marker CD146 along the periphery of the blood vessels captured at 60x magnification using confocal microscopy. Lower panels showed expression of these markers in the longitudinal section of whole pulp tissue.



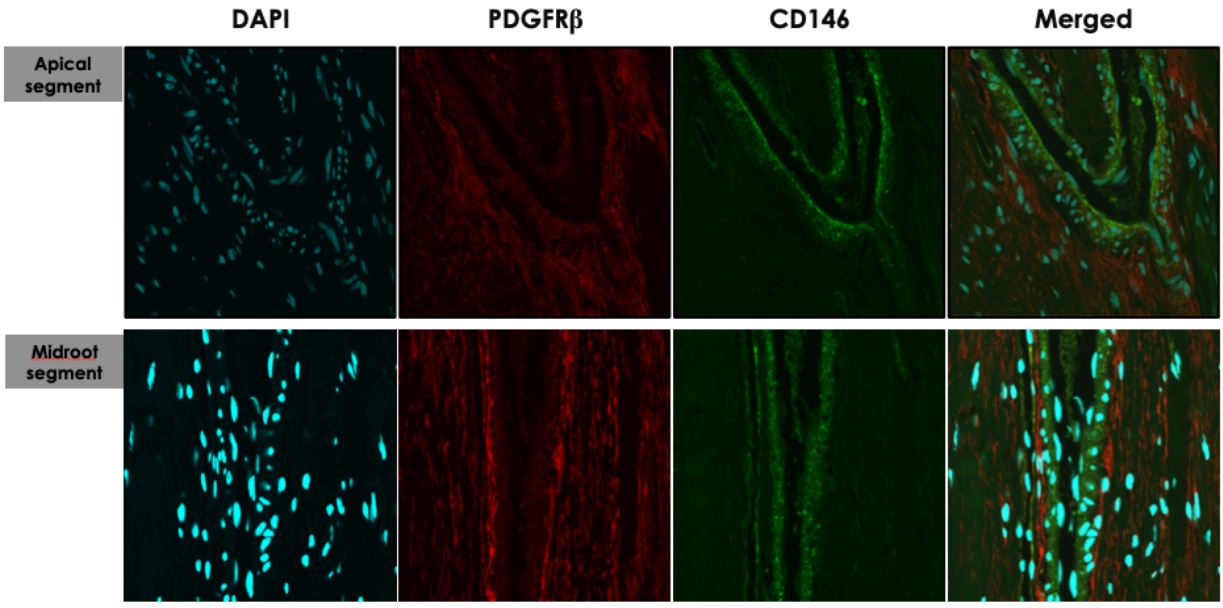
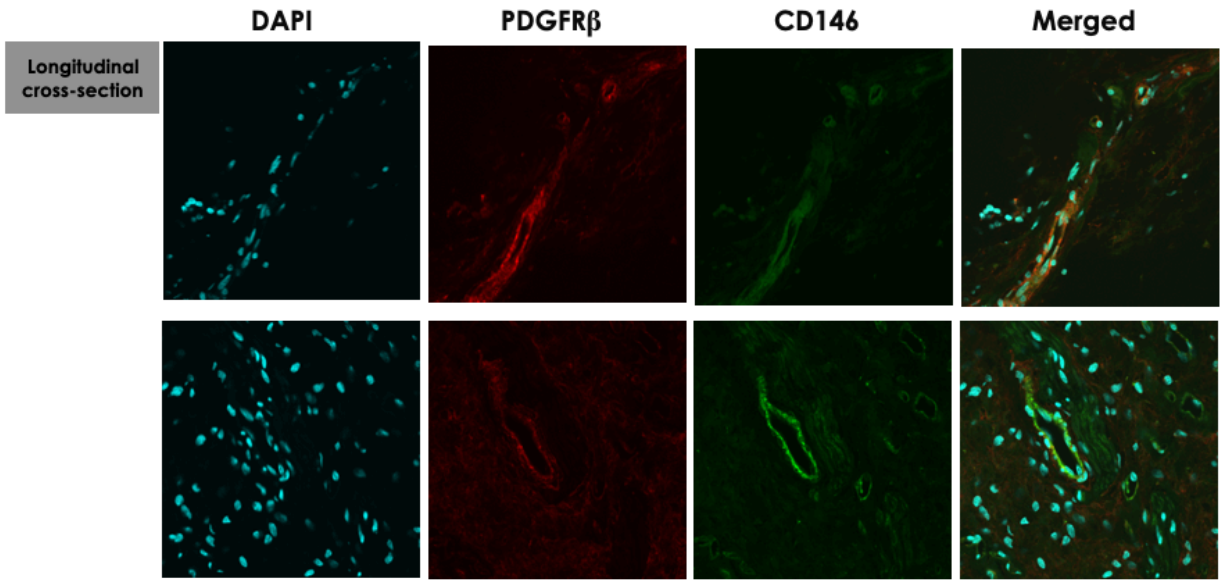
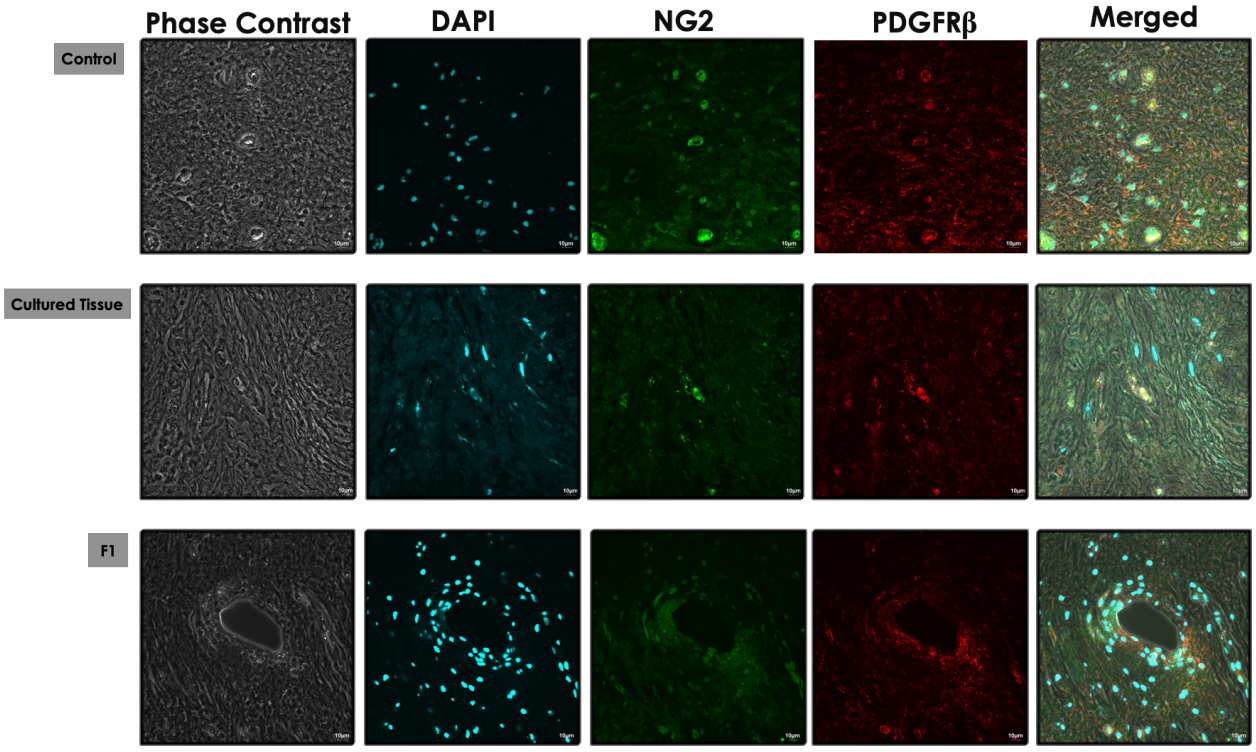


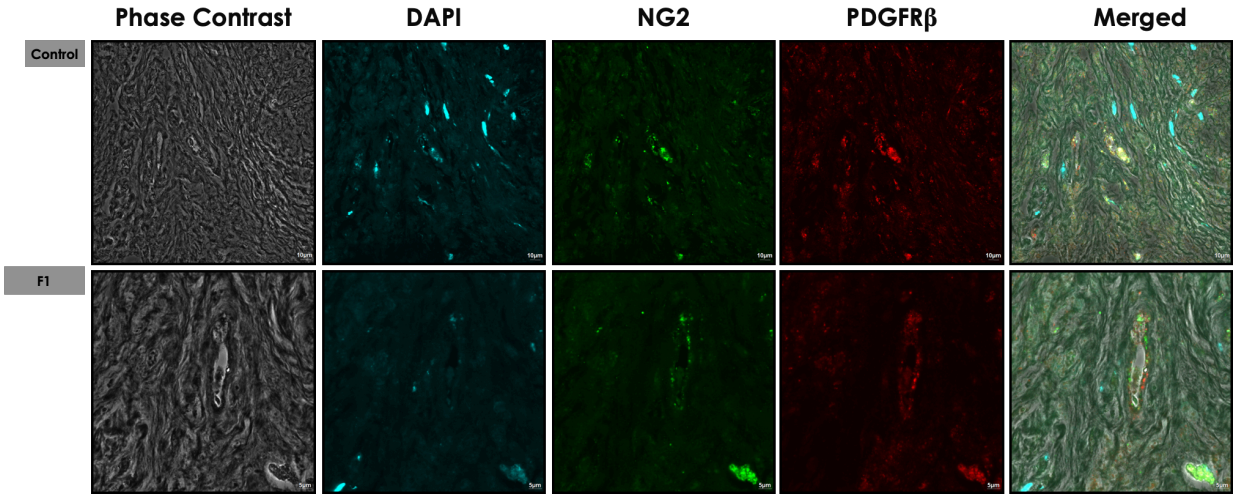
Figure 7. Immunofluorescence microscopy showing pericyte markers.



b) Expression of pericyte marker PDGFRβ and NG2 along the periphery of the blood vessels captured at 60x magnification using confocal microscopy.



c) Cultures tissue to 80% confluency exhibits continued expressing pericyte markers in both groups.



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